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Mapping of the Cellular Immune Responses to Woodchuck Hepatitis Core Antigen Epitopes in Chronically Infected Woodchucks

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T-cell responses to hepatitis B virus nucleocapsid antigens (HBcAg and HBeAg) play an important role in disease outcome in those infected with hepatitis B virus (HBV). The woodchuck is naturally infected in the wild with woodchuck hepatitis virus (WHV), which shows a high degree of genetic homology to HBV and produces a similar pattern of infection in its natural host. Twenty-three overlapping peptides were constructed to cover the entire WHV core region and used to identify immunodominant cellular epitopes in the nucleocapsid antigen using peripheral blood lymphocytes from 12 chronic WHV carrier and 4 uninfected control animals. A peripheral blood lymphocyte response was seen in all of the chronic WHV carrier animals to at least one peptide, and in 8 of the 12 chronic carrier animals a response was observed to 5 common peptides: peptide analogues of amino acids 16–30, 38–52, 50–69, 76–90 and 91–105. Peptide 91–105 produced maximal proliferation in 5 out of 12 infected animals. In addition, a difference in response was observed between wild and laboratory infected animals; the latter appeared to have a lower response to peptides than animals infected in the wild. This study provides evidence that the woodchuck has a population of peripheral blood cells which are sensitised to epitopes within the nucleocapsid protein and provides a basis on which to develop the use of the woodchuck as an immunological model of HBV infection for testing therapeutic means of enhancing this response. ***J. Med. Virol. 52:128–135, 1997.***

KEY WORDS: WHV; cellular immune responses; WHcAg

INTRODUCTION

Both the elimination of hepatitis B virus (HBV) from an infected patient and the pathology of the disease are thought to be immune mediated [Dudley et al., 1972]. Elimination of HBV is usually marked by the loss of the soluble hepatitis B e antigen (HBeAg), which shares the majority of its amino acid sequence with the viral nucleocapsid antigen hepatitis B core antigen (HBcAg) [Ganem and Varmus, 1987]. Using overlapping peptides immunodominant T helper-cell epitopes have been identified within this region [Ferrari et al., 1991; Jung et al., 1995].

During acute HBV infection the T helper cell response to the nucleocapsid antigens is detectable in virtually all patients [Ferrari et al., 1990; Jung et al., 1991]. This is in contrast to patients who are persistently infected. During acute exacerbations of persistent type B hepatitis, T cell responses to these antigens are increased [Tsai et al., 1992; Marinos et al., 1995] and this coincides in some patients with seroconversion to anti-HBe positivity [Marinos et al., 1995]. This data and the observation that recovery from HBV infection is associated with the presence of the MHC Class II allele DRB1*1302 [Thursz et al., 1995] suggest that the T helper cell response plays an important role in the outcome of HBV infection.

Woodchuck hepatitis B virus (WHV) infection in the American woodchuck (*Marmota monax*) is a naturally occurring model system for HBV infection in humans. Both viruses have a similar morphological and antigenic structure [Summers et al., 1979; Werner et al., 1979; Feitelson et al., 1981], and there is considerable homology between their genomes [Cumming et al., 1980; Galibert et al., 1982]. Both viruses cause similar pathological lesions and have a disease spectrum which ranges from acute self-resolving infection to chronic active persistant infection. The woodchuck, therefore, is a good animal model for the investigation of the role of the immune response to these viruses and in determining outcome following therapeutic intervention.

In order to improve the usefulness of this model, we have investigated the proliferative immune response to the WHV core antigen (WHcAg) using a panel of over-

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lapping synthetic peptides covering this region in 12 chronic carriers of WHV and 4 uninfected control animals.

MATERIALS AND METHODS

Woodchucks

4 uninfected (W859, W32, W867, W939) and 12 adult chronic carriers of WHV, of which 9 were wild caught (W507, W518, W520, W526, W527, W540, W556, W936, W937) and 3 were laboratory infected (W360, W363, W414), were obtained from Northeastern Wildlife (S. Plymouth, NY) and maintained at our animal facilities in standard laboratory steel cages. The laboratory infected animals were infected with the WHV 7 strain.

Animals were anaesthetized, and blood samples, taken into heparin (10U/ml), were obtained by venepuncture from the antecubital vein of the hind legs. Blood was also taken for serum, which was separated after centrifugation at 1000 rpm for 4 minutes. Three animals (W859, W936, W937) were killed by exsanguination and their spleens excised. All procedures were carried out in accordance with Home Office guidelines.

WHV Status of the Chronic Carriers

WHV DNA was detected by dot blot hybridisation. Briefly, 100ul of serum was digested using 20ul 100mg/ml pronase, 1.7ul 25% SDS, 2.5ul 0.8mg/ml t-RNA, and 75.8ul buffer solution (40mM Tris, 0.3M NaCl, 20mM EDTA, pH 8.0) at 37°C overnight and extracted with an equal volume of phenol equilibrated with buffer solution. After denaturing with 100ul of 0.15M NaOH/1.5M NaCl and neutralisation with 100ul of 0.15M Tris-HCI pH 7/1.5M NaCl, samples were spotted onto a nitrocellulose filter and baked for 2 hours at 80°C. Filters were incubated for 16 hours with a radiolabelled probe prepared by labelling 120ng of linearized WHV DNA with ^{32}P-dCTP by nick translation (Amersham, UK). Specific activities of probes were between 4–8 × 10^8 cpm/ug. The filter was washed and air dried before exposure to preflashed film overnight at −70°C.

WHeAg was measured using the Enzymun Test for anti-HBe and HBeAg (Boehringer Mannheim, Germany); results were expressed in Paul Erlich units. This was done in comparison to the standard included in the kit. This is a human antigen standard calibrated by the Paul Erlich Institute (1 unit is equivalent to 1 ng protein). Gamma GT (GGT) enzyme levels were measured using the Reflotron system (Boehringer Mannhein, Germany). In addition uninfected animals were tested for anti-WHc using the Enzymun test for anti-HBc (Boehringer Mannheim, Germany).

Isolation of Lymphocytes From Blood and Spleens

Spleens were teased apart with sterile needles, washed once in RPMI 1640 medium, and resuspended at 1 × 10^6/ml in Clicks complete medium [Clicks Extra High Amino Acid medium (Irvine Scientific, CA), 0.5% normal woodchuck serum (WCS), 2mM glutamine, 2 × 10^{-5} M 2-mercaptoethanol (2-ME)]. Blood was diluted in RPMI 1640 medium (1:3) and layered onto Ficoll-Paque (Pharmacia, UK). After centrifugation (1000 rpm for 30 minutes), PBMCs were removed from the interface and washed 3 times in RPMI 1640 medium before resuspension in Clicks complete medium at 1 × 10^6 cells/ml. The recovery of lymphocytes was greater than 95%.

Synthetic Peptides

WHV 8 [Girones et al., 1989] is a cloned variant of the WHV genome found in infected woodchucks and shares approximately 65% sequence homology with the human HBV genome. It was found to be 3323 base pairs long, and the nucleotide sequence varied from 4 other WHV genomes by 0.4–3.1% [Girones et al., 1989; Cohen et al., 1988]. Laboratory infected animals were infected with the WHV 7 strain, which differs at two amino acids in the core region [Cohen et al., 1988]. There is a glycine-to-valine change at amino acid 123 and an additional arginine at amino acid 152. The wild infected animals came from an area where both WHV 8 and WHV 7 infect the population. This sequence was used to construct 23 peptides, overlapping by 8 amino acids, covering the entire WHV 8 core region, which were synthesized by Cambridge Research Biochemicals (Zeneca Inc., UK). They varied in length from 12 to 20 amino acid residues to include some of the same epitopes as those found to be cell epitopes in HBV. The peptides, numbered consecutively 1 to 23, were as follows beginning at the first methionine of the core region: 1–15, 8–22, 16–30, 23–37, 31–45, 38–52, 46–60, 50–69, 61–75, 68–82, 76–90, 83–97, 91–105, 98–112, 106–120, 113–127, 121–135, 128–142, 136–150, 143–157, 151–165, 158–172, 166–178.

Lymphocyte Proliferation Assay

The optimum culture conditions which would support proliferation of woodchuck PBMCs were determined by measuring the dose response to phytohaemagglutinin (PHA) in 9 adult woodchucks of which 5 were chronic carriers of WHV and 4 were uninfected animals. Maximum proliferation was observed when PBMC were cultured in Clicks medium (Irvine Scientific) supplemented with 0.5% woodchuck serum, 2mM glutamine, and 2 × 10^{-5}M 2-mercaptoethanol (2-ME).

The kinetics of the response were determined in response to the mitogen PHA and to WHcAg peptides over a time period of 3 to 9 days.

Cell proliferation was assessed by counting total cells per well under the microscope and by measuring the incorporation of methyl-^{3}H thymidine, 5- ^{3}H uridine, or ^{3}H bromodeoxyuridine (Amersham International, UK). The length of time of the pulse with the radiolabels was varied from 6 to 24 hours and labels of specific activity ranging from 2 to 27Ci/mmol were used.

Once the conditions for the measurement of the proliferative response were established, the dose response to peptides (concentration range 1–10ug/ml) and to PHA (concentration range 1–10ug/ml) was tested in all

of the adult woodchucks used in the study. Cells were also cultured without antigen in triplicate in each assay. All microcultures were set up in triplicate in 96-well round bottomed plates (Nunc, Denmark) at a concentration of 2×10^5 cells/well and incubated at 37°C in 5% CO_2. The final volume in the well was 200ul.

After the radiolabelling of the culture, the cells were harvested onto glass fibre filters (Packard Instruments, UK) and the filters were air dried for 2 hours and counted using a direct beta counter (Matrix 9600, Packard Instruments, Groningen, Netherlands). The standard error between replicates was calculated; results were used only when this was less than 20%. The results were expressed as a stimulation index (SI), calculated as the ratio between mean counts per minute (cpm) obtained in the presence of peptide and the mean cpm obtained in the absence of peptide. SIs of greater than 3.5 were considered significant indicators of proliferation. This figure was calculated from the mean of the stimulation indices obtained against all the peptides in the uninfected animals plus 2 times the standard deviation from the mean.

RESULTS

Cross Reactivity of WHV Antigens in Assays for HBV Antigens

The use of Enzymun tests (Boehringer Mannheim) for HBeAg detection to test for WHeAg was assessed initially in 5 uninfected animals who had not been given WHV and who were WHV DNA negative, and in 3 chronic carrier animals who were WHV DNA positive. Neat serum from these animals was tested. The uninfected animals were negative (mean OD, 0.0132) compared to the kit negative control (mean OD, 0.015) and the chronic carriers were strongly positive for antigen (OD > 3.0); the mean OD of the kit positive control was 2.423. Sera from 15 chronic carriers were subsequently tested and found to be positive, and when serially diluted and tested for antigen, the mean titre was 1/64. Six chronic carriers were tested weekly over 8 weeks and remained positive throughout that time.

The initial tests for anti-WHc using the anti-HBc Enzymun test were done on neat serum from 2 uninfected WHV-DNA negative animals, 2 animals who had received an inoculum of WHV but had not become WHV-DNA positive, and 3 WHV-DNA positive chronic carriers. The 2 uninfected animals were negative for antibody (OD 1.005, 0.328) with the cutoff OD 0.256, the 3 chronic carriers were positive (OD 0.045, 0.081, 0.212), and the 2 animals who were inoculated but did not become infected were borderline (OD 0.257, 0.240). A further 3 uninfected animals were negative for antibody and 13 chronic carriers were all positive.

Serology of Uninfected Animals

W859 was positive for anti-WHc, while W939, W32, and W867 were negative. All 4 were negative for WHBeAg and were WHV DNA negative. W859 had been inoculated with WHV-DNA but had not become WHV-DNA or WHeAg positive.

Chronic Carrier Status of the Woodchucks

All WHV chronic carrier animals used were WHV DNA and WHeAg positive (Table I). Laboratory infected animals were infected at birth and at the time of testing were 8 months old. The length of time wild caught woodchucks have harboured the virus is unknown and therefore the effect of this on the proliferative response to nucleocapsid antigens cannot be determined. The WHV DNA levels in laboratory infected and wild caught animals were comparable, although the WHeAg levels were lower in laboratory infected animals. The measurement of GGT levels was used to monitor the development of hepatocellular carcinoma (HCC). Five animals had elevated levels above 2.8 IU/L, and of these, 2 were very high (W936 and W937). These 2 animals also had high WHV DNA and WHeAg levels.

Lymphocyte Proliferation Assay

Initial experiments to measure the incorporation of methyl tritiated thymidine of specific activity 25Ci/mmol or 5Ci/mmol and pulse times of 6, 18, and 24 hours into cells stimulated with PHA for 4 days gave low or undetectable levels of incorporation of radioactivity despite the fact that blast cells were visible under the microscope. Direct comparison between the number of cells in mitogen stimulated wells and those in unstimulated wells showed a large increase in stimulated wells. In one representative experiment, the mean number of cells in wells stimulated with 1ug/ml PHA for 4 days was 13.83×10^5 cells/well, whereas in unstimulated wells the mean number of cells was 1.67×10^5 cells/well. In the same experiment, incorporation of tritiated uridine gave a stimulation index of 8.27, which correlates well with the cell number increase.

Tritiated thymidine of the lower specific activity was incorporated more efficiently over an 18-hour period than the higher specific activity material over a 6-hour period. In a comparison between incorporation of tritiated thymidine (5Ci/mmol) and tritiated uridine (27Ci/mmol) over a 6-hour period, a greater incorporation of uridine was detected (Fig. 1A), although the dose response curves were similar. The proliferation of human cells from a normal subject in response to PHA could be detected using uridine in a dose-related manner, but the maximum SI was much lower than with thymidine (Fig. 1B). The incorporation of the thymidine analogue bromodeoxyuridine by woodchuck cells stimulated with PHA was also compared to the incorporation of uridine. Bromodeoxyuridine was incorporated confirming that the cells do proliferate, although the stimulation index was greater in cells pulsed with uridine. (Fig. 1C).

The kinetics of the proliferative response to both the mitogen PHA and to antigen derived peptides was found to be the same giving a maximal response at 4 days.

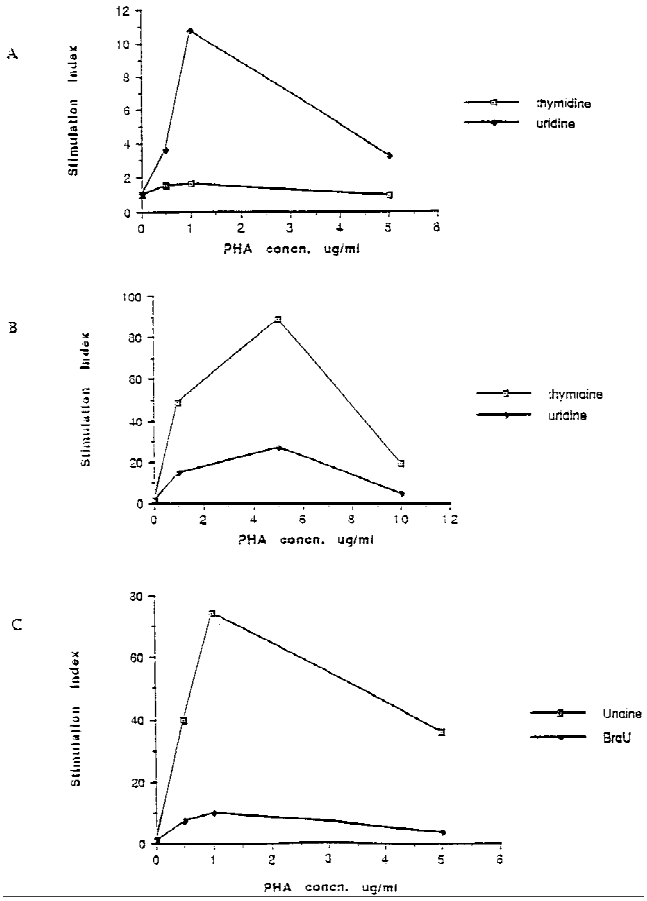

Fig. 1. Graphical illustration comparing the incorporation of tritiated thymidine and uridine by **(A)** woodchuck PBMC stimulated with PHA, and **(B)** human PBMC. The incorporation of bromodeoxyuridine and uridine by woodchuck PBMC was also compared **(C).**

TABLE I. Summary of Data Indicating the Serological and Biochemical Status of Chronically Infected Animals

Animal number	WHV DNA (pg/ml)	WHeAg (P. Erlich units)	GGT Level (IU/L)
Naturally infected			
W507	103.21	75.20	<2.8
W518	50.18	62.47	<2.8
W520	11.30	68.82	10.90
W526	82.75	75.92	<2.8
W527	146.94	77.86	<2.8
W540	260.13	71.82	14.6
W556	54.01	75.45	4.94
W936	11065	583.04	424
W937	13800	658.56	68.7
Experimentally infected			
W360	119.22	13.26	<2.8
W363	139.37	30.57	<2.8
W414	104.55	20.62	<2.8

The Proliferative Response to Peptides

The dose response of PBMC to each of the peptides in each of the animals was determined. The results shown are a comparison of the response to each of the peptides at a concentration of 5ug/ml, since this concentration gave maximal stimulation for all of the peptides (Table II). Therefore the stimulation indices measured at this concentration can be used to assess the immunogenicity of the peptides in the individual animals.

PBMC from all chronic carriers responded to at least one peptide, and one animal, W937, which had been infected in the wild, responded to all peptides (Table II). The number of animals whose PBMCs responded to each peptide is illustrated in Figure 2. Peptides 16–30, 38–52, 50–69, 76–90, and 91–105 induced a proliferative response in the majority of infected animals (8 out of 12 animals). These results demonstrate that nearly all of the core sequence is immunogenic.

The results were also analysed to assess the magnitude of the PBMC response to each of the peptides. The dose responses to each of the peptides were compared and the peptide to which each animal responded with the highest stimulation index (SI) was recorded. The greatest number of animals responded maximally to peptide 91–105 (Fig. 3). Five out of 12 chronic carrier animals responded maximally to this peptide; one of these was laboratory infected (W414) and the other 4 animals were wild caught. Two animals responded maximally to peptide 38–52 and a further 2 animals to peptide 143–157.

The laboratory infected animals responded poorly to the peptides. Animals W360 and W363 responded to 3 peptides and W414 to only 1 peptide. Of the 4 uninfected animals, W867 had stimulation indices slightly greater than 3.5 in response to 4 peptides. W939 and W32 did not respond to any of the peptides, and W859 had slightly raised stimulation indices in response to 2 peptides. All of these responses were minimal.

DISCUSSION

In humans the HBV nucleocapsid antigens induce an MHC class II restricted T-cell proliferative response which is associated with viral elimination [Jung et al., 1995; Tsai et al., 1992; Marinos et al., 1995]. The ability of both HBcAg and WHcAg to stimulate a protective immune response against HBV and WHV infection, in chimpanzees and woodchucks, respectively [Roos et al., 1989; Murray et al., 1984, 1987; Iwarson et al., 1985], indicates the importance of these antigens during the course of infection.

In order to assess cellular immune responses in woodchucks, the optimum conditions had to be determined. The choice of radiolabel proved to be the largest obstacle in defining the experimental conditions of a cell culture system for woodchuck lymphocytes. Tritiated thymidine, of different specific activities, was tested at a range of different pulse times, but thymidine uptake was detected only at a very low level. The increased number of cells observed in stimulated wells suggested that the cells were indeed undergoing cell division.

Two early papers show that woodchuck cells are unable to incorporate thymidine efficiently and suggest

TABLE II. Woodchuck Lymphocyte Responses to WHcAg Synthetic Peptides in Uninfected and WHV Chronically Infected Animals

	Stimulation indices: status of woodchucks[a]															
	Uninfected				Naturally infected									Lab. infected		
Peptide	859	32	867	939	507	518	520	526	527	540	556	936	937	360	363	414
1. 1–15	1.29	1.63	0.69	2.52	4.43	1.31	1.55	0.95	0.98	2.99	1.44	2.03	25.61	2.14	2.19	0.76
2. 8–22	1.49	1.70	3.02	2.15	3.96	5.55	0.58	1.64	1.46	8.30	4.64	1.86	19.60	0.53	1.61	1.01
3. 16–30	4.27	2.18	1.54	2.21	6.71	2.92	4.57	0.89	2.84	22.79	10.64	7.39	37.29	5.14	3.72	1.42
4. 23–37	3.91	3.19	0.91	1.58	4.26	1.71	4.19	1.48	2.14	9.39	13.91	4.13	34.36	7.09	2.80	1.65
5. 31–45	3.31	2.80	1.32	1.28	1.92	1.46	4.94	1.27	2.30	13.90	16.09	4.14	29.76	2.43	4.51	1.41
6. 38–52	2.37	1.21	1.21	1.86	2.10	4.15	11.85	3.25	6.92	17.37	15.15	4.07	33.51	2.30	2.14	0.56
7. 46–60	1.16	1.61	0.87	2.52	16.17	2.32	4.08	1.19	2.16	3.61	12.00	4.91	38.34	2.29	1.71	0.73
8. 50–69	2.5	2.17	0.91	1.47	5.47	2.11	3.88	0.97	2.00	9.53	3.50	6.28	51.23	3.83	3.62	1.22
9. 61–75	1.67	2.23	0.99	1.28	4.75	1.59	4.94	1.18	0.93	2.60	0.61	5.84	51.59	1.51	1.97	0.72
10. 68–82	1.95	1.70	1.33	2.18	8.17	2.59	6.17	1.08	0.99	10.03	3.44	3.21	35.08	2.14	1.77	0.73
11. 76–90	1.49	1.78	1.35	2.06	6.05	2.09	10.64	2.33	1.00	14.65	3.45	19.74	46.76	3.49	1.18	3.46
12. 83–97	1.29	1.67	3.60	1.37	1.87	2.51	4.01	1.76	0.53	7.98	1.86	2.08	34.15	3.10	0.57	2.24
13. 91–105	3.03	2.47	4.82	2.43	24.30	17.42	3.86	4.61	0.89	17.05	1.84	35.99	66.45	1.83	1.43	3.82
14. 98–112	2.06	2.40	3.20	1.90	5.06	2.28	3.84	1.14	0.98	8.76	2.51	2.79	21.91	2.75	1.21	1.68
15. 106–120	1.64	1.75	2.89	0.98	4.59	2.33	4.84	1.05	1.47	9.57	2.13	2.62	25.58	1.94	0.90	1.51
16. 113–127	2.13	1.53	3.03	2.00	4.79	1.80	3.60	0.99	1.08	30.37	1.80	4.50	37.71	1.62	0.57	2.51
17. 121–135	1.67	1.26	2.85	1.44	1.44	12.51	6.02	0.93	1.01	15.14	2.62	2.85	29.73	0.87	0.88	2.44
18. 128–142	1.41	0.80	4.04	0.76	1.76	2.89	3.96	1.10	0.67	11.37	2.37	2.33	25.97	0.84	0.45	2.30
19. 136–150	1.69	1.46	3.87	0.91	4.88	2.13	3.61	0.72	0.53	11.36	2.61	2.50	26.37	0.80	0.71	2.35
20. 143–157	2.45	1.34	2.73	1.00	8.23	17.42	6.56	5.19	1.28	34.91	2.92	3.94	37.86	0.59	1.96	2.44
21. 151–165	2.75	1.88	3.02	2.72	7.42	4.00	0.79	2.89	1.00	9.45	7.02	2.31	25.48	0.99	1.01	0.53
22. 158–172	2.57	2.75	2.81	2.11	6.63	4.04	0.76	2.41	1.32	13.88	6.06	2.31	21.31	0.65	2.09	0.82
23. 166–178	1.63	2.03	1.81	2.43	1.48	5.16	0.53	1.07	2.25	15.27	12.98	2.52	28.50	0.75	1.40	0.66

[a]Stimulation indices greater than 3.5 are considered positive. They are underlined.

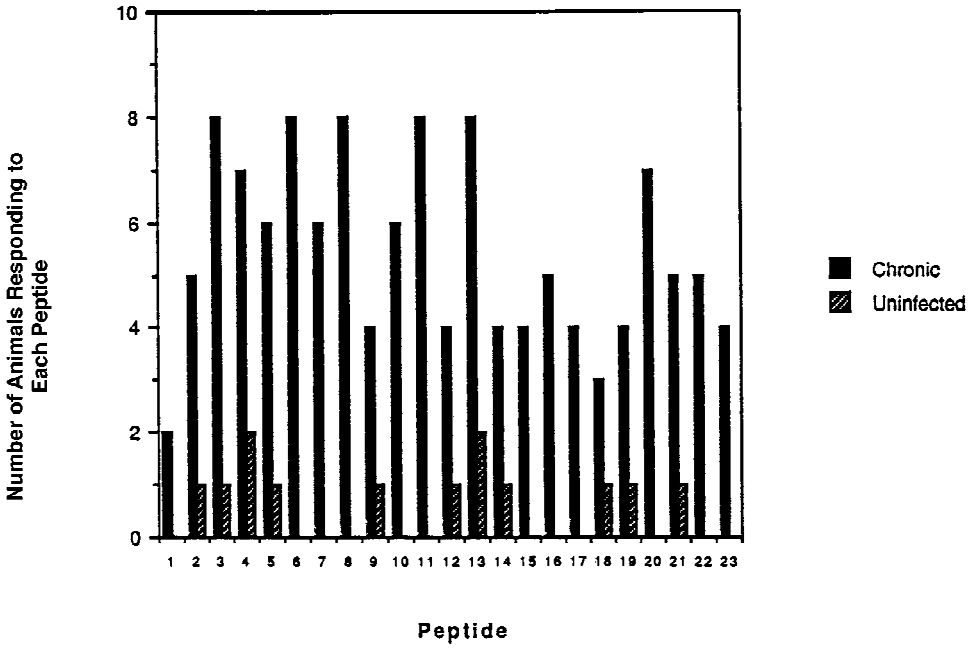

Fig. 2. The number of animals responding to each of the WHcAg peptides from 12 chronic carrier and 4 uninfected woodchucks.

that woodchucks may have a relative deficiency of thymidine kinase in their cells [Adelstein et al., 1964, 1968]. Thymidine kinase is necessary for the phosphorylation of the thymidine nucleoside, enabling its incorporation into DNA via the salvage pathway [Grav and Smellie, 1963, 1965].

Tritiated uridine uptake by lymphocytes in mice, rats, and humans has been well documented [Howard et al., 1972; Gutman and Weissman, 1975; Scott and Josephs, 1975]. It is taken up by blast cells produced by stimulation with PHA in larger amounts than by small lymphocytes [Hayhoe and Quaglino, 1965], and it is not completely specific for RNA but can also be converted to DNA precursors [Comings, 1966]. The tritiated label is retained when uridine is converted to cytidine, which is converted via the diphosphorylated molecule into de-

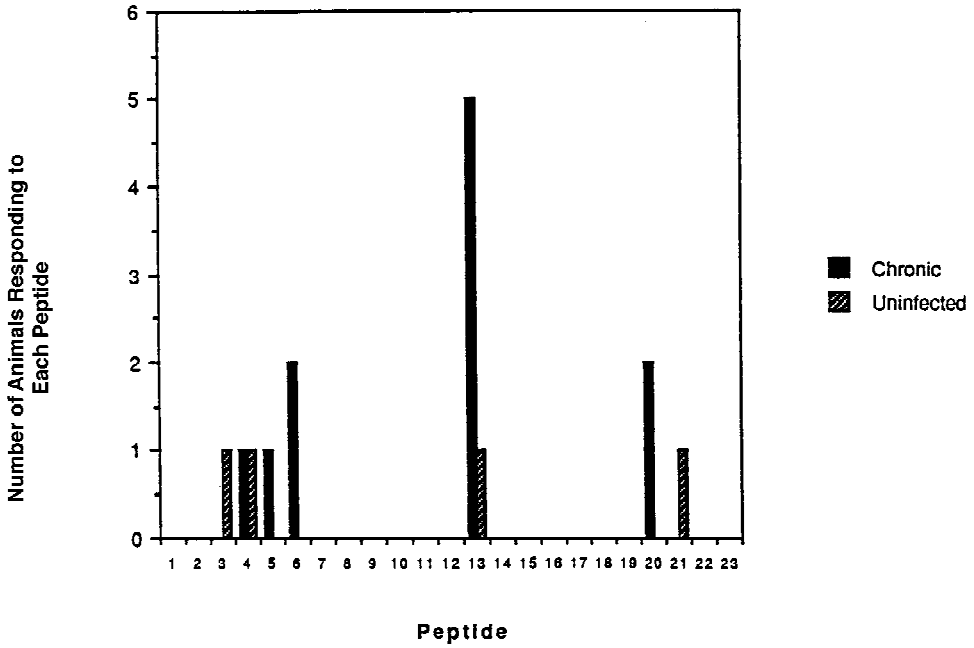

Fig. 3. The magnitude of the PBMC response to each of the peptides by each of the infected animals was analysed and the numbers of animals responding with their greatest magnitude to a given peptides were plotted.

oxyribosylated cytidine [Comings, 1966]. Uridine incorporation was found to be very efficient in woodchuck PBLs and its magnitude was similar to the increase in cell number. The kinetics of the uptake mirrored that of an increase in the incorporation of the thymidine analogue bromodeoxyuridine. A comparison of the dose response to mitogen in humans as measured by the incorporation of thymidine or uridine further demonstrated that uridine was taken up in proportion to the level of proliferation of the cells. In woodchucks, uridine is a useful tool to circumvent the poor incorporation of thymidine. The lack of incorporation of thymidine confirms findings by another group [Cote and Gerin, 1987] who used uridine or adenosine to label proliferating cells.

The results of this study show that all 12 chronically infected animals, whether infected in the wild or in the laboratory, responded to at least 1 nucleocapsid peptide, and 1 animal responded to all 23 peptides. Six of the naturally infected animals responded to a large number of peptides. A similar wide range of T-cell responses to HBcAg peptides was seen in patients with both acute and chronic HBV infection using peptides of a similar size [Jung et al., 1995]. The broad response may be explained by the size of the peptides, which are large and potentially contain more than 1 epitope. The animals are an outbred population and would all be able to present peptides in the context of more than one class II MHC molecule.

Of the 5 animals that responded to fewer than 5 peptides, 3 were laboratory infected. The 2 amino acid differences between WHV 7 and WHV 8 would not account for any lack of response to the other peptides. In the laboratory infected group, 8 months had elapsed since infection, whereas the length of time for which the wild caught animals had been infected was unknown. Both W936 and W937 had developed HCC. This suggests that they had harboured the virus for a period of 2 to 3 years [Popper et al., 1987]. Both of these animals had significant cellular responses to a large number of peptides; W936 responded to 12 peptides, and W937 responded to all 23 peptides. These results may indicate that the longer the animal has harboured the virus, the more diffuse the specificity of the response to the nucleocapsid antigens.

The 8 chronically infected animals who mounted a significant proliferative response to a minimum of 8 peptides, W507, W518, W520, W527, W540, W556, W936, and W937, all responded to peptide 91–105 except W556. Indeed, in 5 chronically infected animals, the greatest proliferation in response to any of the peptides was induced in response to peptide 91–105. Together these results suggest that this sequence of 15 amino acids is an important epitope in woodchucks. It does not coincide precisely with epitopes reported to be important in humans, although it does partly overlap peptide 81–105, which was found to be immunogenic in 37% of acute and chronically infected patients who responded to core antigen [Jung et al., 1995].

T lymphocytes recognise peptides derived from processed antigens bound to MHC molecules, and their recognition is restricted by the binding of the peptide to one or a few alleles in an allele specific conformation. However, some peptides have also been shown to bind promiscuously to a large number of MHC class II molecules, some of them in similar conformations, making these peptides widely immunogenic [Panina-Bordignon et al., 1989]. Experiments with truncated and substi-

tuted versions of the peptide 91-105 would distinguish between the possibility that this long peptide contains several epitopes which bind to different alleles or 1 peptide epitope which binds promiscuously to a large number of alleles.

Although the uninfected animals responded to some of the peptides, the stimulation indices observed were very low in comparison to those observed in animals who were chronically infected. It is possible that these animals may have been exposed to WHV and mounted a T-cell response in the absence of antibody seroconversion or that these low level responses are nonspecific.

Two groups have identified T-cell epitopes to which patients with acute disease are sensitised and that are dominant irrespective of the HLA haplotype. One group [Ferrari et al., 1991] showed that 90% of patients with acute HBV responded to a peptide of amino acids 50–69. Others have identified peptides 1–25 and 61–85, in which 52 and 59% of patients responded, respectively [Jung et al., 1995]. No reagents for T-cell surface markers in the woodchuck are yet available, but by analogy with other mammalian species, it seems likely that the observed proliferation of PBMC is in the main proliferation of T cells. In addition, although nothing is yet known about the MHC of the woodchuck, these animals are an outbred population, and it seems likely that peptide 91–105 represents an epitope which is presented by most MHC Class II glycoproteins.

Although during acute disease there is a marked T-cell response to nucleocapsid antigens (45% of patients respond), this is much less frequent in chronically infected patients, in whom 13% respond [Jung et al., 1995]. In acute exacerbations of chronic disease and during HBeAg/anti-HBe seroconversion, an increased T-cell responsiveness is observed [Tsai et al., 1992]. In comparison, the 66% of responders in the group of chronically infected woodchucks studied is much higher than the response seen in patients with chronic HBV. It has been suggested that chronic disease in a woodchuck is more similar to active disease in the human [Ponzetto et al., 1984]. If this is the case, the more vigorous response observed here in chronically infected woodchucks than that seen in chronically infected patients is to be expected.

In conclusion, we have demonstrated a strong proliferative PBL response in animals chronically infected with WHV to WHcAg synthetic peptides. Activation of CD4+ T lymphocytes by nucleocapsid epitopes is thought to be necessary for viral elimination [Tsai et al., 1992] and a therapeutic means of enhancing this immune response may be of value in the treatment of chronic hepatitis. The epitope mapping of this response will enhance the usefulness of the woodchuck model in testing new treatments for chronic hepatitis.

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